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THE EFFECTS OF EXTRACELLULAR pH AND BUFFER CONCENTRATION ON THE EFFLUX OF LACTATE FROM FROG SARTORIUS MUSCLE

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SUMMARY

1. The rate of efflux of lactate from isolated frog sartorius muscles is measured with a superfusion technique. Efflux curves are followed after raising the internal lactate level of the muscles by repetitive electrical stimulation over a 200 sec period.

2. With an external pH of 7·0 or below the measured efflux rates following stimulation reach 100–150 n-mole/g.min. Increasing the pH of the superfusion fluid to 8·0 results in a two or threefold increase in the peak efflux rate. The effect is independent of the buffer system used and occurs fairly rapidly when the pH of the superfusion fluid is changed. This suggests that the effect of pH on lactate efflux is extracellular.

3. The increase in efflux rate due to an increase in pH is dependent on buffer concentration. This fact together with measurements of surface pH changes in muscles following arrest of superfusion indicates that a pH gradient exists through the muscle thickness during lactate efflux.

4. The low lactate efflux rate seen at a low buffer concentration (1 mM) is reduced to an even lower level by depolarization with potassium sulphate suggesting a membrane potential dependent component. At pH 8·0 with a high buffer concentration (25 mM) potassium sulphate only reduces efflux rate slightly.

The observations are interpreted as indicating that a fraction of lactate lost is in the form of undissociated acid and that this fraction increases with increasing external pH.

5. Conditions which favour loss of hydrogen ions and lactate from muscle are also associated with improved recovery of twitch tension.

INTRODUCTION

Lactate formed in muscle fibres during activity diffuses into the extracellular space and appears in the blood. This has been assumed by some

workers to be a rapid simple diffusion process (Margaria, Cerretelli, diPrampero, Massari & Torelli, 1963). The early work of Eggleton, Eggleton & Hill (1928) suggested that the diffusion may not always be rapid and that the apparent diffusion coefficient for lactate is reduced in fatigue. Karlsson (1971) and Diamant, Karlsson & Saltin (1968), using muscle biopsies, have shown that fairly large concentration differences can exist between lactate in muscles and blood after strenuous exercise.

We have previously found that muscles equilibrated in Ringer solution with a high bicarbonate concentration (25 mM) show a more rapid rate of lactate loss than muscles equilibrated in low bicarbonate (1 mM) (Mainwood, Worsley-Brown & Paterson, 1972). This seemed to suggest an extracellular rate limiting step in lactate removal from isolated muscles. Since these experiments were carried out, Isutzu (1972) reported that intracellular buffering power of isolated muscles can be considerably changed following equilibration in Ringer solution containing different bicarbonate concentrations.

These observations lead to a number of questions: are the rate limiting steps in lactate efflux from muscles intracellular or extracellular as suggested by Eggleton *et al.* (1928)? Is the effect of bicarbonate on lactate efflux due to a changed extracellular or intracellular pH or is it a specific effect of bicarbonate? Does most of the lactate lost from the muscle pass through the muscle fibre membrane in the undissociated acid form in spite of the fact that the pK_a value for lactate must be normally at least three pH units below intracellular pH?

We have attempted to answer some of these questions by measuring the rate of lactate efflux from isolated superfused sartorius muscles into different external solutions. The muscles were stimulated electrically by a standard procedure in order to elevate muscle lactate levels to about 25 μmole/g. Changes in twitch tension associated with fatigue and recovery were monitored during the lactate efflux measurements. Net hydrogen ion efflux rates have also been measured in modified Ringer solutions with different buffer systems.

METHODS

Sartorius muscle preparation

Paired sartorius muscles were used in most experiments, taken from frogs (*R. pipiens*) of about 3 in. body length. All frogs were injected with D-tubocurarine (3 mg), 30 min before the preparation, to avoid muscle activity during preparation and provide a more uniform biochemical starting point (Marechal & Mommaerts, 1963). After removal of the skin from the hind limbs, the resting length of the muscles was measured. In most experiments, blood was washed from the legs by cannulation of the abdominal aorta and injection of about 10 ml. Ringer solution. The pelvic girdle was split in the mid line with a razor blade and the sartorius muscles carefully dissected out with a thread tied round the distal tendon. After

being cleaned, the pelvic bone was pierced by a number 22 hypodermic needle to provide a hole for the hook to attach it to the stimulation frame.

Most muscles used weighed 80–110 mg and were less than 0·8 mm thick, though weights outside this range were sometimes used. It is important to restrict muscles to a fairly narrow range of muscle sizes since the time required for diffusion into and wash out of the extracellular spaces is dependent on muscle size. Muscles with a mean thickness of 0·8 mm or less have an 80% extracellular washout time of 5–10 min in this system (Lucier & Mainwood, 1972). In spite of standardizing procedures, both the amount of lactate formed and the shape of the efflux curve vary. Variation is much less between paired muscles so that the effect of variables was tested between pairs wherever possible.

Mounting and stimulation. The muscles were attached to a frame between parallel platinum stimulating wires and equilibrated in a large volume of oxygenated Ringer solution for 30–45 min. At the beginning of the experiment, the muscles in the frames were blotted with filter paper and mounted in the plexiglass chambers kept at 20° C, through which gas mixtures at the same temperature and vapour pressure as the Ringer solution were passed (Fig. 1). The hooks through the pelvic bones were attached to transducers (Grass FT 03) by light silver chains, and extended to a length 1 mm longer than the resting length. The muscles were equilibrated in the chamber for about 1 hr before the start of the experiment.

The response of the muscle to single shocks at 5 min intervals was recorded during the period of equilibration in the chamber and values of the rate of resting lactate efflux measured. In some experiments the tension developed in response to brief tetanic stimulation (0·18 sec at 80/sec) was also measured. The procedure for stimulation to fatigue was designed to increase muscle lactate levels to 25 μmole/g (in O_2). This can be achieved by stimulation with 200 maximal shocks at 2/sec. During this stimulation period twitch tension and tetanus tension fall to about 20% of the rested state value. Recovery of isometric tension in response to single stimuli or brief tetani is slow and takes about an hour in solutions with high buffer concentrations. Recovery is incomplete with low buffer levels.

Solutions

The 'basic' bicarbonate Ringer solution used for superfusion contained Na^+ 120, K^+ 2·5, Ca^{2+} 1·8, HCO_3^- 25, Cl^- 101·1 (all in mM). Chloride ions were substituted for bicarbonate when the bicarbonate concentration was changed. When other buffer solutions were used, 0·5 M stock solutions were made up and titrated to the appropriate pH. The sodium and chloride ion concentration in the Ringer solution were adjusted so that the final solution always had a calculated osmolarity of 250 m-osmole.

Superfusion. The superfusion fluid was delivered through PE 190 polyethylene tubing from a Harvard infusion pump (Model 940) at flow rates in the range of 13–55 μl./min. The tubing just touched the top inner surface of the muscle below the pelvic bone and flowed down over the whole inner surface to the distal tendon. The effluent was collected in a Teflon cup under the muscle and withdrawn at intervals by suction (Fig. 1). The gas mixture was equilibrated by passing it through three scintered glass aerators in series into solutions of the same osmolarity as the Ringer. The gas flow rate was maintained at 60 ml./min. Under these conditions we found negligible water loss from the muscle (determined by weighing after 2 hr in the chamber) or from the superfusion fluid as measured by concentration changes of radioactive inulin or sorbitol while passing over the muscle at flow rates of 13 μl./min (Lucier & Mainwood, 1972).

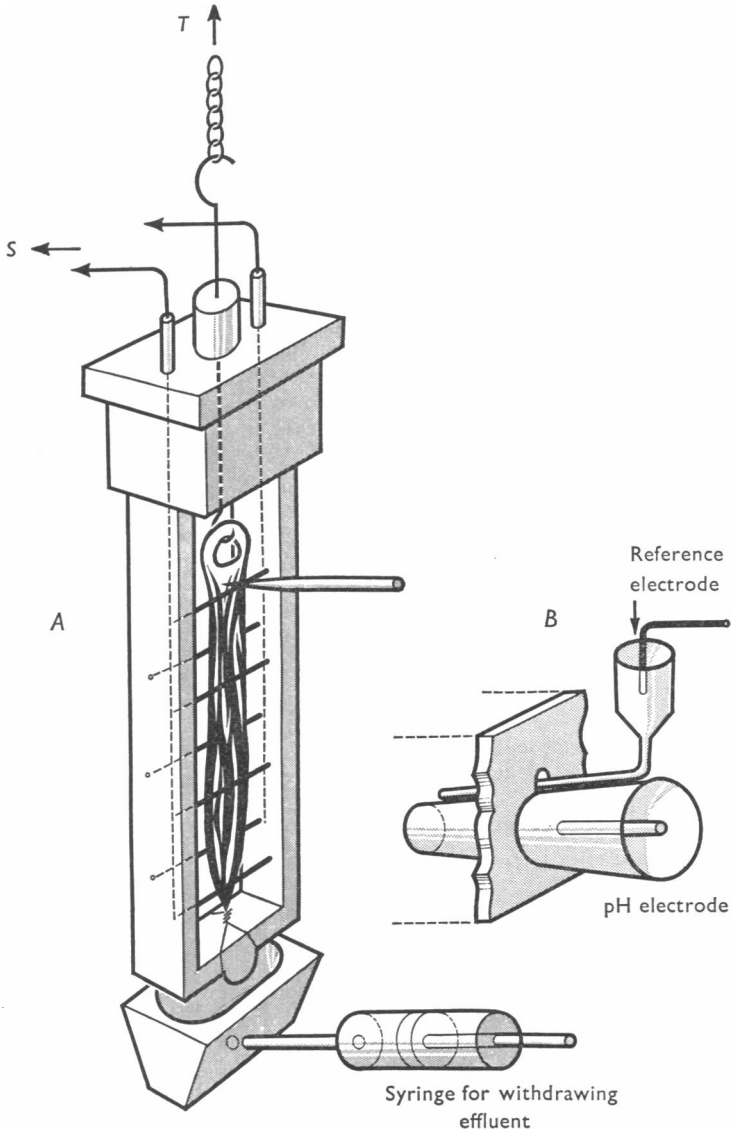

Fig. 1. Diagram of apparatus showing (*A*) a sartorius muscle in the stimulating frame. *S*: stimulator; *T*: tension transducer. *B*: arrangement of surface pH electrode in front panel.

Freezing and extraction

At the end of the efflux experiments, muscles were quickly frozen for biochemical assay. The strain gauge was unhooked and the stimulating frame with the muscle attached withdrawn from the chamber. The frame was inverted in a stand so that

the muscle could be drawn out from between the electrodes after cutting through the tendon at the pelvic end. As soon as the muscle was pulled clear of the electrodes it was frozen between aluminium blocks precooled in liquid nitrogen. The muscle was then stored at $-70°$ C until it was extracted for biochemical assay.

Muscles were extracted for assay as follows: the frozen muscle was wrapped in a square of aluminium foil precooled in liquid nitrogen. The wrapped muscle was then placed in a rectangular recess in a precooled stainless steel block. A cold steel piston fitting into the recess was struck several times with a hammer so that the muscle was powdered. The foil wrap stops the loss of muscle fragments and at the same time prevents condensation and ice formation on the muscle. The powder was tipped into a weighed 'Sorval' pot containing 1 ml. frozen 6% perchloric acid. After reweighing, a further 3 ml. cold perchloric acid was added and the mixture stirred in ice. The slurry was homogenized and allowed to stand 10 min on ice. After centrifuging for 10 min at 10,000 rev/min in the cold, 3 ml. of the supernatant was neutralized with 2·5 M potassium carbonate and the perchlorate precipitated. After centrifuging again for 5 min at 10,000 rev/min the supernatant was decanted and kept on ice for biochemical assay.

Biochemical assay. Lactate was assayed in both the muscle extract and muscle effluent. This was done by measuring the reduction of DPN (Boehringer-Mannheim) in the presence of lactate dehydrogenase (LDH) (Worthington Biochemicals Ltd) by following the increase in optical density at 340 nm (37° C) with a Unicam SP 800 spectrophotometer. The method described by Olson (1962) was used in which semicarbazide was used to 'trap' pyruvate formed in the reaction.

20 μl. DPN (40 mg/ml.) and 10 μl. LDH (20 mg/ml.) were pipetted into a quartz cuvette together with 0·9 ml. freshly made buffer with semicarbazide (1·5 g glycine, 2·23 g semicarbazide HCl dissolved in 80 ml. H_2O, and adjusted to pH 10 with NaOH and made up to 100 ml. with distilled water). The optical density at 340 nm was read until steady and then 0·1 ml. of the sample added. The optical density increased and readings were taken every 2 min until it remained constant, which usually took about 20 min. The same volume of distilled water was added to reagents in a parallel cuvette to correct for the dilution effect of the sample. The volume of the sample was sometimes increased to 0·2 ml. for low lactate concentrations.

Net lactate efflux rates were calculated by multiplying the superfusion flow rate by the effluent concentration. Efflux per g was then calculated from the final frozen weight of the muscle.

ATP was measured by the method described by Bergmeyer (1965).

pH and H^+ efflux. Measurements of surface pH were carried out in some experiments with a flat pH electrode (Fig. 1*B*). The pH of effluent samples was measured with a Radiometer blood pH capillary electrode. The fall in pH is a result of the increased P_{CO_2} around the muscle fibres (Dubuisson, 1954) as well as the H^+ efflux from the muscle. The effect of P_{CO_2} changes in non-bicarbonate buffers may be eliminated by equilibration with CO_2 free gas, but this takes a long time with alkaline solutions in the region of pH 8·0. Small water losses from the effluent samples which may occur in this process can then lead to rather large errors in the estimates of H^+ balance.

To overcome this problem the pH of the effluent samples was lowered by adding a known amount of acid and calculating the H^+ balance from the final pH. Prolonged contact with glass of samples having a low buffer concentration resulted in instability and fluctuations of 0·1–0·2 pH units which were unexplained but avoided by collecting the samples in polyethylene tubing. 50 μl. samples of the effluent were acidified by adding 50 μl. of standardized lactic acid and mixing thoroughly. The strength of the acid was previously adjusted to bring the calculated

final pH of the superfusion fluid to about 4·5. The sample now lies in the pH range of the lactate buffer system and the net H^+ change in the effluent can be readily calculated from the final pH reading (pH_2) according to the formula:

$$X = \frac{L}{1+10^{pH_2-pK_L}} + \frac{B}{1+10^{pH_2-pK_B}} + 10^{-pH_2}V - (A+S),$$

where X = the net hydrogen ion gain by the effluent sample from the muscle (n-mole).

L = total lactate content of the sample (n-mole).

B = total buffer content ($B^- + BH$) of the sample (n-mole).

V = volume of sample + additions (nl.).

A = amount of buffer originally in the acid form (BH), in n-mole.

pK_B = the pK_a of the buffer in the superfusion fluid.

pK_L = the pK_a of lactate (3·7).

S = acid added to the sample (n-mole of H^+) to reduce to the required pH range.

pH readings were taken in triplicate and a set of standards prepared by adding known amounts of lactic acid to samples of the superfusion fluid. It was found convenient to programme eqn. (1) (on a Wang programmable calculator) so that values for X could be read off directly for any given values of pH_2 and L, the other values remaining constant for any one experiment. The method was regularly checked by measurement of the pH of a parallel set of standards with each experiment.

RESULTS

Lactate content and efflux rate in CO_2–HCO_3^- buffer system

A series of eleven resting sartorius muscles in Ringer solution buffered by 1 mM-HCO_3^-, 1 % CO_2 (pH 6·51) were frozen in the resting state and found to have a lactate content of $1·8 \pm 1·3$ μmole/g. The lactate efflux rate was in the range 10–20 n-mole/g.min in these muscles. Following stimulation to fatigue (200 sec, 2/sec) the lactate content measured in a further nine muscles increased to $25·9 \pm 6·5$ μmole/g. The efflux rate is increased to 100–150 n-mole/g.min immediately after fatigue. This rate of efflux remains constant or declines very slowly, although the lactate content decreases to about 10 μmole/g.min in the 50 min period following stimulation to fatigue.

The rate of lactate efflux during the post-stimulation period may be increased two- to threefold by increasing the external bicarbonate level to 25 m-equiv/l. (pH 7·9). The experiment illustrated in Fig. 2 shows twitch tension response and lactate efflux from a pair of sartorius muscles from the same frog, subject to the same stimulation procedure. The bicarbonate content of the superfusion fluid in one of the pair was increased from 1 to 25 mM, 20 min after the period of repetitive stimulation and the increase in lactate efflux is evident in the first 5 min collection period after the change, and is associated with an increase in twitch tension. The rate of efflux is not measurably greater in muscles presoaked for an hour or more in 25 mM bicarbonate.

In order to obtain more quantitative information on the dependence of lactate efflux on external bicarbonate and pH, conditions were standardized as closely as possible. A series of six pairs of sartorius muscles from the same batch of frogs were all stimulated in 1 mM bicarbonate. Twenty minutes after the beginning of the 200 sec stimulation period each muscle of each pair was switched to a Ringer solution containing a different bicarbonate concentration in a random series. The mean rate of lactate efflux in the interval before the change was 150 n-mole/g. min.

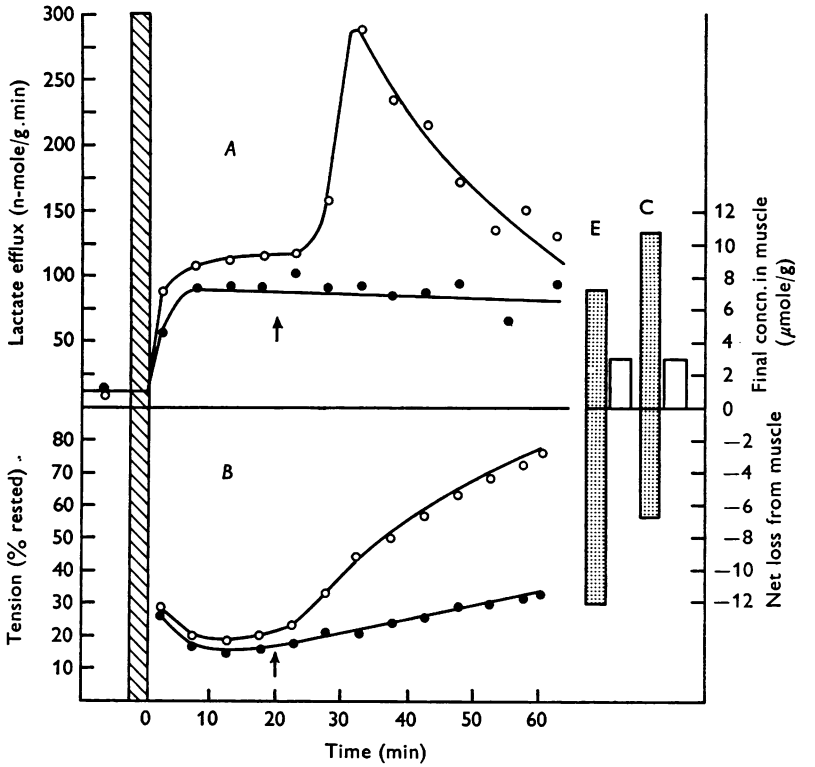

Fig. 2. Lactate efflux and twitch tension during recovery from fatigue. *A*: lactate efflux rate. Vertical hatched column on left represents the stimulation period of 200 sec at 2/sec. One muscle (○) fatigued in 1 mM-HCO_3^-, pH 6·5. At ↑, superfusion fluid switched to 25 mM-HCO_3^-, pH 7·9. Control paired muscle (●) superfused with 1 mM-HCO_3^- throughout. Columns on right show: lactate remaining in muscle (stippled column above zero line); net lactate loss from muscle (stippled column below line) and ATP content of muscle at the end of the recovery period (open columns). Left columns (E) are experimental muscle in which bicarbonate was increased, right hand columns (C) control muscle. Lower graph *B*, shows corresponding twitch tension in control (●) and experimental (○) muscles. Abscissa: time after the end of stimulation.

When the muscles were switched to a higher bicarbonate level the rate of lactate efflux reached a peak in the second 5 min interval after the change in each case. The mean lactate efflux values taken at this time were plotted against pH or bicarbonate concentration. It can be seen that the curve relating lactate efflux to bicarbonate concentration is steep over the range 1–10 mM or over the pH range 6·5–7·5 (Fig. 3). From 10 to 25 mM there is comparatively little further increase in lactate efflux rate. The recovery of twitch tension (as a percentage of the pre-fatigue level) increases more linearly as a function of bicarbonate concentration up to 25 mM.

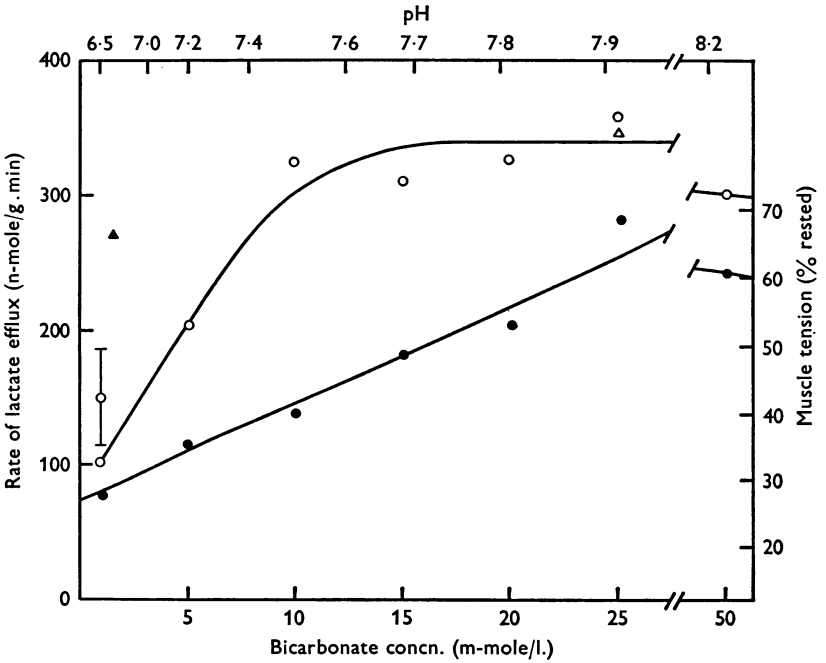

Fig. 3. Summary of results of lactate efflux and twitch tension in seven pairs of muscles. Each muscle was stimulated to fatigue in 1 mM-HCO_3^- buffer and lactate efflux was measured in the following 10 min period (mean and S.D. indicated by open circle and vertical bars on left). Each muscle was then switched randomly to superfusion fluids with different bicarbonate concentrations between 1 and 50 mM. After 10 min, lactate efflux rate (○) was again measured and the mean of paired observations plotted against bicarbonate concentration (lower scale) or pH (upper scale). Twitch tension at the end of a 40 min recovery period is also shown (●). One pair of muscles was stimulated in 25 mM-HCO_3^- and the external pH then adjusted to 6·6 by increasing P_{CO_2} (▲) while the control (△) remained at a P_{CO_2} of 8 torr at pH 7·9.

In order to distinguish between the effects of bicarbonate concentration and pH, P_{CO_2} was varied at a constant bicarbonate concentration. In the experiment illustrated in Fig. 4 one muscle was equilibrated with CO_2 at 8 torr throughout the efflux period. The other was switched to a gas

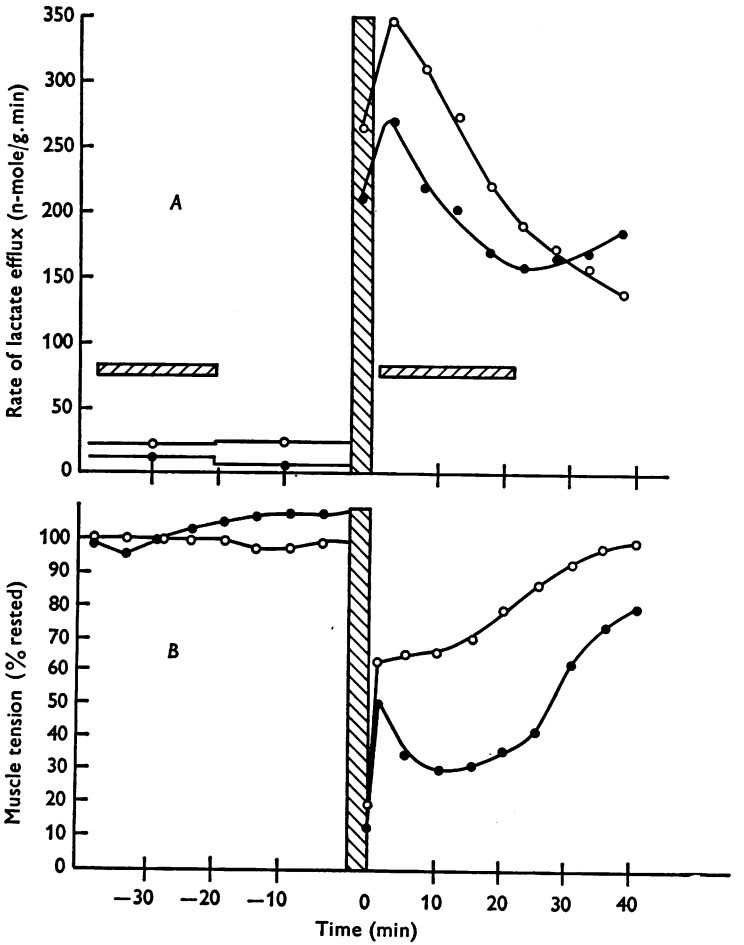

Fig. 4. The effect of increasing P_{CO_2} on twitch tension and lactate efflux in resting and fatigued muscles. Upper graphs *A*, lactate efflux in paired sartorius muscles. Vertical hatched column: stimulation for 200 sec. Horizontal hatched bars indicate duration of exposure of experimental muscle (●) to a P_{CO_2} of 150 torr (pH 6·6) (normal level, 8 torr). Control muscle (○) at a P_{CO_2} of 8 torr throughout, both muscles superfused with 25 mM-HCO_3^- Ringer (pH 7·9). Lower graph *B* shows twitch tension as a percentage of initial tension. Abscissa: to the right of the vertical hatched column, time after the end of stimulation. To the left of the vertical hatched column, time before stimulation.

phase with CO_2 at 150 torr immediately after stimulation which reduced the extracellular pH from 7·91 to 6·60. This would at the same time reduce intracellular pH by about one unit (Isutzu, 1972). As a result there was a large decline in twitch tension and some depression of the lactate efflux rate. On return to low P_{CO_2} the lactate efflux rate increases to a level above that of the control muscle. Although the increase in P_{CO_2} reduces lactate efflux the rate is still about twice as great as the efflux rate from muscles in a superfusion fluid of the same pH containing a low bicarbonate concentration (Fig. 3).

While external pH seems one important factor in determining efflux rate, buffer concentration may also play a part. The question arises as to whether a specific effect of bicarbonate ions is involved.

If the rate limiting step in the efflux of lactate involved the reaction of hydrogen ions with bicarbonate to form CO_2 in the extracellular fluid it seemed possible that the reaction may be speeded up by the presence of carbonic anhydrase. To test this possibility, muscles were carefully washed free of blood before stimulation and lactate efflux measured in paired muscles, one with and one without added carbonic anhydrase. Carbonic anhydrase (1 mg/ml.) added to the perfusion fluid caused no noticeable difference in the efflux rates at either 1 or 25 mM bicarbonate concentrations.

With a bicarbonate buffer system it is not possible to alter external pH independently of external bicarbonate concentration without at the same time affecting intracellular pH as a result of the increased P_{CO_2}. To avoid this complication, non-bicarbonate buffer systems were substituted. It was also possible that a non-specific bicarbonate effect due to the increased level of extracellular indiffusible anions or reduction of chloride ions could be eliminated in this way. In order to test these possibilities a cationic buffer system, Tris (hydroxymethyl) aminomethane was used.

The effect of varying both the concentration and pH of Tris buffer on lactate efflux is shown in Fig. 5. Efflux rates into 25 mM Tris at pH 8·0 are very similar to the rates measured in 25 mM bicarbonate at the same pH. Much lower efflux rates are seen when either the external pH is reduced to 7·0 while maintaining the same Tris concentration or when the buffer concentration is reduced at the same pH. It thus appears that both external pH and external buffer concentration are important in determining lactate efflux rate. Since Tris itself is a poor buffer at pH 7·0, this test is complicated since the actual buffering power is lowered by lowering the pH. A buffer system with a pK value closer to 7·0 would be preferable. Phosphate has a suitable pK value but some spontaneous activity was found to occur in muscles equilibrated in solutions containing high phosphate concentrations.

Three other buffer systems with pK values close to 7·0 have been tried, imidazole (pK_a 7·09), 2-*N*-morpholino propane sulphonic acid (MOPS) (pK_a 7·10) and N-2-hydroxyethyl piperazine N-2-ethane sulphonic acid (HEPES) (pK_a 7·48). The pK_a values are those given by Murphy & Koss (1968)

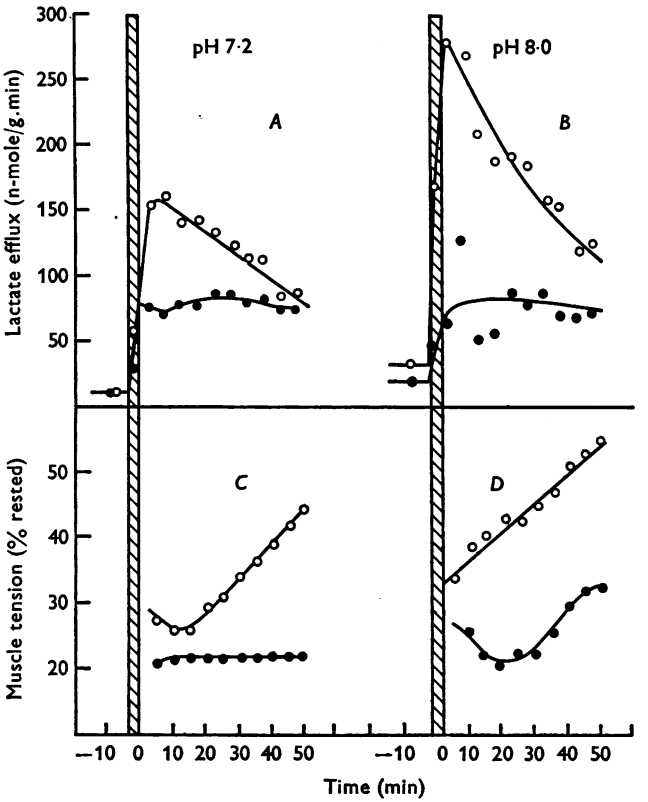

Fig. 5. Lactate efflux and twitch tension following fatigue in Tris buffer. *A*: lactate efflux in a pair of sartorius muscles at pH 7·0. Hatched column indicates 200 sec stimulation period in (○) 25 mM buffer and (●) 1 mM buffer. *B*: pH 8·0; (○) 25 mM (●) 1 mM. *C* and *D*: twitch tension recovery in the corresponding pairs of muscles. Abscissa: time after the end of stimulation.

and were also checked by direct titration in buffer-free Ringer solution. Experiments with these systems (Fig. 6) give similar results to those seen with Tris (Fig. 5). Imidazole was found to be a less satisfactory buffer than the other two since muscle tension showed only poor recovery after fatigue in 25 mM imidazole as compared with the other four buffers. The resting lactate efflux in imidazole also increased to about 3 times the value found with the other four buffer systems. At high buffer

concentrations efflux rate falls as the external pH is reduced from 8·0 to 6·5. Low buffer concentrations at pH 8·0 fail to support high lactate efflux rates even though the pH of the effluent is maintained within about 0·2 pH units of the superfusion fluid entering the chamber. This

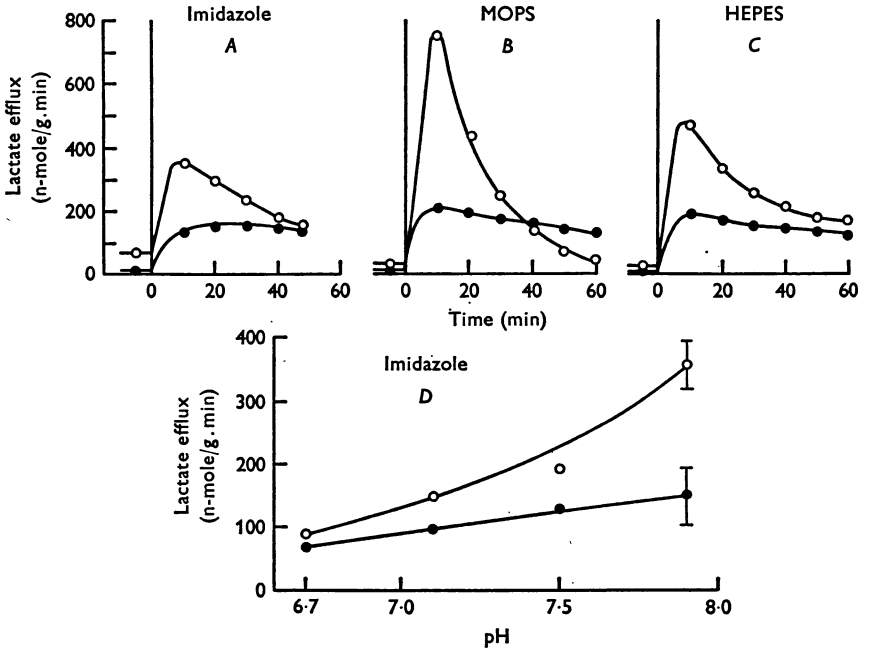

Fig. 6. The effect of pH on lactate efflux using different buffer systems. *A*, *B* and *C*: lactate efflux curves into 25 mM buffer solutions. Vertical line: 200 sec stimulation period. *A*: imidazole buffer, pH 7·90 (○) and 6·80 (●). *B*: MOPS, pH 8·00 (○) and 7·00 (●), *C*; HEPES, pH 8·10 (○) and 6·97 (●), all in 100% oxygen. *D*: the effect of buffer concentration (imidazole) and pH on lactate efflux (○) 25 mM and (●) 1 mM. The vertical bar at the right (pH 7·9), represents the standard deviation of peak efflux of a set of six pairs of muscles. At pH 6·7 the point represents the mean of two pairs and intermediate points represent single pairs of muscles.

suggests that a pH gradient exists at low extracellular buffer concentrations due to diffusion within the muscle itself so that the deeper fibres are at a lower pH than those at the surface.

Evidence for the existence of such a gradient is provided by measurements of muscle surface pH following arrest of superfusion. In a resting muscle using a CO_2:bicarbonate buffer very little change in surface pH is observed when superfusion is stopped. In fatigued muscles surface

pH falls rapidly when perfusion is stopped (Fig. 7). At least part of this fall is due to accumulation of CO_2 under the electrode. This is clearly seen by the rise in pH of the fluid film on the electrode when the electrode is withdrawn from the muscle surface and the fluid re-equilibrates with the CO_2 in the gas phase. When this cycle is repeated several times the pH rise following withdrawal becomes smaller and smaller so that the bicarbonate concentration itself in the extracellular fluid must have decreased.

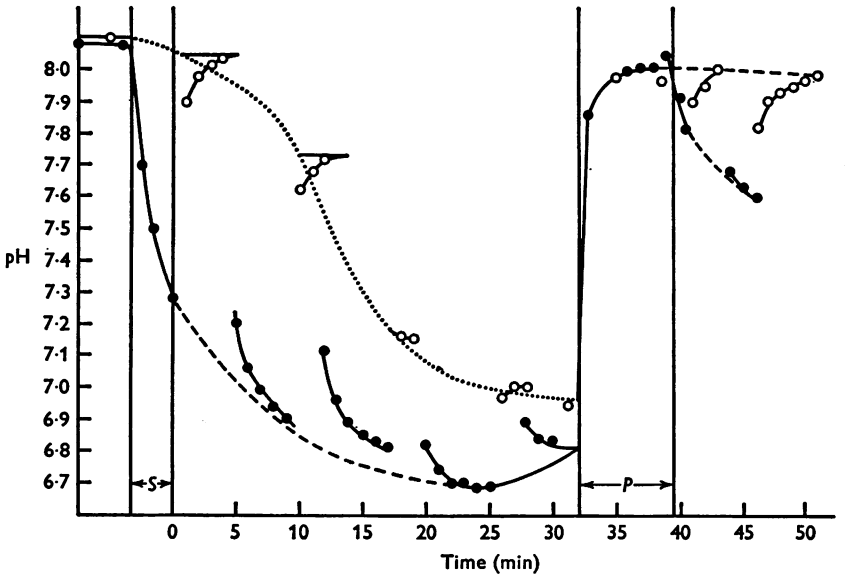

Fig. 7. Surface pH measurements in frog sartorius muscle following superfusion with 25 mM-HCO_3^-–Ringer in 1 % CO_2, 99 % O_2. Measurements were taken with the electrode pressed on the muscle surface (●) (see Fig. 1) and lifted just off the surface (○) to allow the surface fluid to equilibrate with the gas phase. From left: surface measurements following arrest of superfusion in resting muscle. During *S*, muscle stimulated with 400 shocks. During *P*, muscle re-perfused with 25 mM-HCO_3^-. Perfusion stopped again after *P*.

This rather qualitative demonstration of extracellular bicarbonate loss is consistent with the idea that lactate does leave the muscle in the undissociated acid form. If this is so one would expect efflux rate to be independent of transmembrane electrical potential. The effect of membrane potential changes on lactate efflux was investigated by depolarizing the membrane with K_2SO_4 (74 mM) while measuring lactate efflux (Fig. 8). Depolarization may also cause a further small increase in lactate content

of the fatigued muscle though the evidence suggests that glycolysis is largely suppressed at this time so that the change due to depolarization would be small. This is supported by measurements of the lactate content of paired muscles after fatigue. If one of the pair is depolarized, its lactate content is only slightly higher than that in the fatigued, non-depolarized counterpart (Fig. 8).

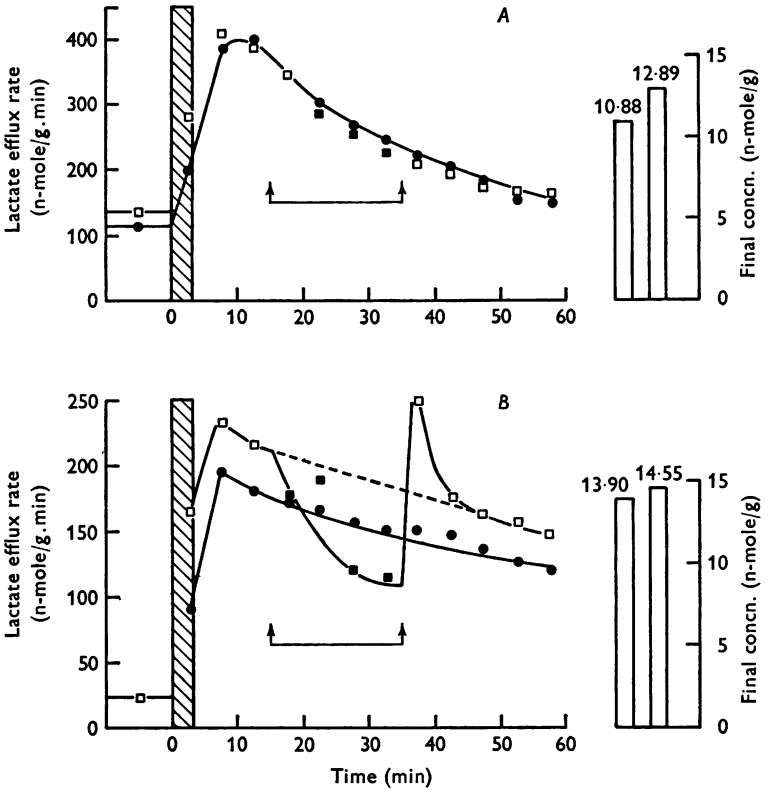

Fig. 8. Lactate efflux and the effect of depolarization with potassium sulphate. *A*: lactate efflux in 25 mM imidazole, pH 8·0. Experimental muscle superfused with normal Ringer (□) and stimulated for 200 sec (vertical hatched column). Between arrows the muscle is superfused with isotonic K_2SO_4 in 25 mM imidazole (■). The paired control (●) is stimulated in normal 25 mM imidazole Ringer throughout. *B*: as in *A*, except the buffer concentration is 1 mM throughout.

Depolarization has only a very small effect on lactate efflux when the extracellular pH and buffer concentration are high. However, there is a marked fall in efflux rate when the muscle superfused with low buffer concentration is depolarized. Fig. 8*A* shows the efflux rate for a pair

of muscles fatigued in 25 mM imidazole buffer at pH 7·95. 15 min after fatigue one of the pair is depolarized with potassium sulphate. In the succeeding 20 min, the lactate efflux rate is consistently lower than that in the control non-depolarized muscle but only by 5–10 %. While this effect is small it seems to indicate a real effect of depolarization on lactate efflux since it returns to reach a level slightly above that in the control level after the depolarizing solution is washed out. At this time the lactate content of the muscle is some 10–20 % above that in the control.

In Fig. 8*B* the lactate efflux rates of a pair of muscles in 1 mM imidazole is shown. Here depolarization has a much clearer effect and lactate efflux is reduced by about 40 % below the interpolated efflux values as a result of depolarization. There is a 'rebound' effect as a result of repolarizing the muscle when lactate efflux reaches a second peak.

Since an increase in P_{CO_2} is effective in lowering intracellular pH, the transmembrane pH gradient should be considerably greater when a given extracellular pH is reached in the presence of CO_2 as compared with a CO_2-free system. This should favour the efflux of the undissociated acid. In order to do this, while at the same time maintaining a high buffer concentration (which is not possible with a CO_2 bicarbonate system alone) a non-bicarbonate buffer system was used in which the pH was adjusted either by equilibration with CO_2 or by HCl and an oxygen gas phase. One muscle of a pair was switched from the CO_2-free system to a CO_2-buffered system at the same pH while lactate efflux was being measured. The change to CO_2 resulted in an increase in efflux rate of about 60 % compared with the control muscle in a CO_2-free system (Fig. 9).

To investigate further the relationship between lactate and hydrogen ion efflux, the hydrogen ion balance of the effluent was measured. This was done by adjusting the pH of all the effluent samples by adding a known amount of lactic acid and calculating the hydrogen ion balance from the final pH reading as described in the methods section.

The results of one experiment is shown in Fig. 10. While the effluent pH of the resting muscle is just below ($-0{\cdot}02$ pH units) that of the superfusion fluid, this is not due to a resting acid efflux other than CO_2. Titration to pH 4·5 shows in fact that there is a small net gain of titratable base in the effluent.

The results of similar experiments on thirteen muscles are summarized in Table 1. In one experiment (321 *A*, *B*) a pair of muscles was superfused with imidazole buffer at two different concentrations (25 and 1 mM). It is clear that low buffer concentrations result in a drastic reduction in both peak hydrogen ion efflux rate and net efflux during recovery.

With MOPS buffer the ratio of net H^+ efflux to net lactate efflux was never greater than 0·7. In 10 mM buffer, the mean net efflux of H^+ over a 40 min period is about 5·0 μmole/g, as compared to about 8·8 μmole/g of lactate over the same period. In 1 mM buffer the mean lactate efflux is reduced to 3·6 μmole/g but the mean hydrogen ion efflux is only about 0·28 μmole/g. While there is certainly considerable individual variation between muscles, it seems clear that lactate and hydrogen ion efflux become uncoupled at low buffer concentrations.

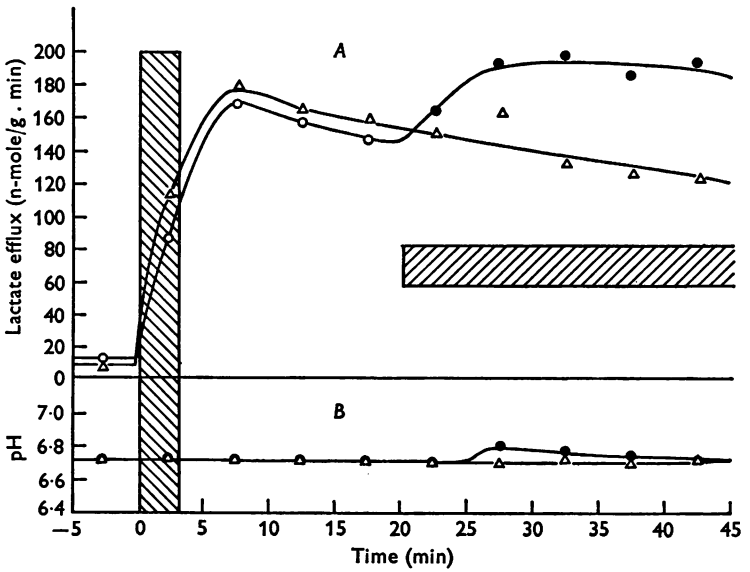

Fig. 9. The effect of CO_2 on lactate efflux at constant external pH. *A*: lactate efflux rate. Vertical hatched bar, stimulation period of 200 sec. Horizontal hatched bar indicates the period in which one muscle was switched from CO_2-free:MOPS buffer solution (○) to 20% CO_2:MOPS buffer system at the same pH (●). Control (△) kept in CO_2-free:MOPS buffer throughout. Lower graph: effluent pH of the control (△) and experimental (●) muscles.

DISCUSSION

The rate of efflux of lactate from fatigued muscle fibres superfused with solutions containing low buffer concentrations quickly reaches a level of 100–150 n-mole/g.min which declines only slowly during the next hour, in spite of the fact that muscle lactate levels fall to less than half the initial value. This suggests that something other than simple diffusion through the extracellular space is limiting the rate of lactate efflux.

When the extracellular buffer concentration is increased from 1 to 25 mM at pH 8·0, the rate of lactate efflux increases quite rapidly to

reach a peak within 5–10 min after the change. With muscles of the size used in these experiments this is the order of time required for ions in the extracellular space to reach a mean value of about 80% of that in the surface solution following an abrupt concentration change (Lucier & Mainwood, 1972). The peak rate of efflux in 25 mM buffer is usually about 300–400 n-mole/g.min and decreases with time in a way which

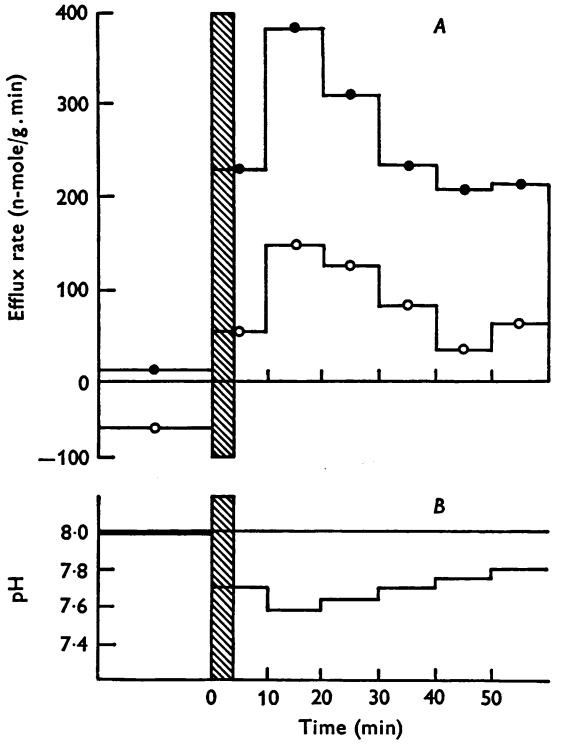

Fig. 10. Net efflux rates of H^+ and lactate from a frog sartorius muscle. *A*: efflux rates, H^+ (○) and lactate (●) before and after stimulation for 200 sec (vertical hatched bar). *B*: pH of effluent. Note the efflux rates in this series of experiments, in which H^+ efflux is measured, are carried out at a perfusion rate of 13 μl./min as compared to 55 μl./min in most other experiments where the effluent pH shift is much smaller. 10 mM-MOPS buffer, pH 8·0.

suggests that the rate limiting step is removed and that the efflux rate is now dependent on the transmembrane lactate concentration gradient. Because of the speed with which the increase in lactate efflux occurs after increasing buffer concentration it seems likely that the action of the buffer is extracellular.

Eggleton *et al.* (1928) observed changes in the apparent permeability

coefficient of lactate in fatigued muscles. They put forward the hypothesis that swelling of the muscle fibres reduced the extracellular space and so limited the diffusion pathways from the muscle in fatigue. Diffusion conditions may have been quite different in their experiments since they used a muscle mass about 50 times greater than that used in the experiments described in this paper. It seems unlikely that reduction of extracellular space plays a significant part in limiting lactate efflux in the sartorius muscle. If so, one would have to suppose that an increase in buffer concentration leads to a rather rapid expansion of extracellular space to account for the increase in lactate efflux.

Karpatkin, Helmreich & Cori (1964) suggested a 'saturation type of efflux mechanism' to explain their observations on stimulated frog sartorius. They estimated a V_{max} for lactate efflux of 0·8 μmole/ml. of intracellular water.min. A carrier type of mechanism is not excluded by our observations but the V_{max} would have to be very sensitive to external pH.

The question of whether lactate leaves the cell as such or as lactic acid is certainly most important from the point of view of hydrogen ion balance within the cell. Loss of lactate ions alone would result in a positive intracellular hydrogen ion balance and yet there would still be no electrochemical gradient to drive hydrogen ions out until the internal pH reaches about 6·0 (Waddell & Bates, 1969). The early work of Berg (1911) suggested that there was an acid efflux from fatigued muscles. The correlation between increased blood lactate levels in exercise and the decrease in blood bicarbonate, shown for instance by Keul, Doll & Deppler (1972), reinforces the idea that hydrogen ions move together with lactate at least in the intact organism.

Our results are not consistent with the view that lactate leaves muscle fibres entirely as lactic acid. With an external buffer concentration of 10 mM at pH 8·0 the net hydrogen ion loss during the 40 min recovery period following stimulation is about half of the net lactate loss (mean 57 %, Table 1). At low external buffer concentrations (1 mM) the hydrogen ion loss is less than 10 % of the total lactate loss. These observations indicate a variable degree of coupling between hydrogen ion and lactate loss depending on the external pH and/or buffer concentration. At low external buffer concentrations a relatively large fraction of the efflux appears to be in the form of lactate ions. One consequence of this is that the net efflux rate should be dependent on transmembrane potential in accordance with the constant field equation.

$$M_L = P_L \frac{VF}{RT}\left[\frac{L_o - L_i \exp - VF/RT}{1 - \exp - VF/RT}\right],$$

where M_L is the net lactate ion efflux rate and P_L is the lactate permeability coefficient and L_o and L_i are the extracellular and intracellular lactate concentrations.

The potential dependent fraction can be approximately estimated from the data shown in Fig. 8. In 8*B*, muscle lactate 30 min after stimulation is estimated to be 20 mM/kg, giving a value of about 30 mM for L_i (correcting for extracellular space and solids but ignoring intracellular binding of water or lactate) L_o is close to 1 mM (based on effluent concentration). If we assume that the fatigued fibres in Ringer solution have a transmembrane potential of -70 mV (Persson, 1963) which is reduced to a value close to zero in isotonic potassium sulphate (Hodgkin & Horowicz, 1959) then the ratio of lactate efflux in potassium sulphate: efflux in Ringer may be calculated. This calculated ratio is 0·32. The observed value based on the interpolated Ringer efflux curve (interrupted line) is about 0·6. This is probably an over-estimate, since depolarization itself appears to cause some further increase in lactate production in the fatigued state. It seems likely that a closer estimate of the lactate efflux ratio is given by comparing the lactate efflux during depolarization with that following return to normal Ringer. In this case the ratio is 0·4 which is consistent with 80–90 % of the efflux being due to lactate ions. On the other hand the decrease in efflux rate due to depolarization at pH 8·0 with high external buffer concentration is barely detectable and the potential dependent fraction cannot be more than 10 % of the total. While the estimates are only approximate they are in reasonable agreement with those obtained by measuring net lactate and hydrogen ion efflux (Table 1).

The rate of loss of undissociated lactic acid appears to depend on the maintenance of a steep transmembrane pH gradient. This is further supported by the increase in efflux rate that can be shown to occur when the intracellular pH is lowered by increasing P_{CO_2} and at the same time maintaining the external pH constant (Fig. 9).

The increase in anion conductance associated with an increase in external pH (Hutter & Warner, 1967; Woodbury & Miles, 1973) may account in part for the increase in lactate efflux resulting from an increase in external pH. One may argue that low buffer concentrations are unable to maintain a high pH in the deeper layers of tissue because of hydrogen ion efflux and therefore lactate efflux is reduced as a result of decreased anion conductance in the deeper layers. Our surface pH measurements add support to this view.

There are, however, two arguments against the observed changes in lactate efflux being entirely due to this mechanism. First, a high external buffer concentration at high pH should favour the efflux of lactate as

TABLE 1. The effects of external buffer concentration on lactate and hydrogen ion efflux from fatigued muscles

(1) Muscle ref.	(2) pH, buffer type	(3) Concentration	(4) Peak H^+ efflux (n-mole/g.min)	(5) Peak lactate efflux (n-mole/g.min)	(6) Net H^+ efflux in 40 min recovery (μmole/g)	(7) Net lactate efflux (μmole/g)	Ratio net H^+ / Net lactate
321 A	8·06 Imidazole	25 mM	453	291	14·4	10·81	1·33
321 B	8·00 Imidazole	1 mM	37	141	1·22	5·20	0·23
514	8·00 MOPS	25 mM	234	396	8·39	12·23	0·69
503	7·99 MOPS	25 mM	121	263	3·67	8·38	0·44
530	7·99 MOPS	10 mM	116	165	3·95	5·75	0·69
523	8·00 MOPS	10 mM	148	381	3·78	11·50	0·33
525	7·97 MOPS	10 mM	116	219	4·07	6·96	0·58
1101	7·92 MOPS	10 mM	263	334	8·03	10·98	0·67
	Mean (10 mM-MOPS)		161	275	4·96	8·80	0·57
1010	8·00 MOPS	1 mM	−2·4	70	−0·14	1·50	−0·09
1016	8·15 MOPS	1 mM	17·0	130	0·33	4·68	0·07
1019	7·95 MOPS	1 mM	33·2	156	1·00	5·18	0·19
1024 A	7·94 MOPS	1 mM	3·7	114	0·40	4·05	0·10
1024 B	8·04 MOPS	1 mM	0·4	74	−0·18	2·65	−0·07
	Mean (1 mM-MOPS)		10·4	108·8	0·28	3·61	0·04

dissociated ions. A large part of the efflux should therefore be potential dependent. In fact just the opposite is the case, depolarization at high external buffer concentrations has very little effect on lactate efflux. Secondly the ratio of lactate:hydrogen ion efflux may be expected to increase as buffer concentration is raised. Once again this is contrary to the observed effects.

We are left with the interpretation that the efflux of undissociated acid can in fact make a considerable contribution to net lactate efflux but only when external pH and buffer concentration are high. At pH 7·0 or at buffer concentrations which are less than about 10 mM, lactate ion efflux approaches the net rate of lactate efflux, i.e. about 100 n-mole/g.hr. This may be related to the prolonged net potassium efflux of 70–80 n-mole/g.min which we have observed following stimulation to fatigue in muscles bathed in low buffer concentrations (Mainwood & Lucier, 1972).

Since the pK of the lactate:lactic acid system is about 3·7 (Lockwood, Yoder & Zienty, 1965) only about 0·05 % of the intracellular lactate can be in the undissociated form in the resting state. The net increase in intracellular hydrogen ions during fatigue of about 18 μmole/g (Mainwood *et al.* 1972) should lead to a fall of about 1 unit in intracellular pH. This is greater than the fall predicted in a similar situation by Piiper (1971) in rat

muscle. The reason for this is that the buffering power of frog muscle (Isutzu, 1972), seems considerably less than that of rat (Heisler & Piiper, 1971) and the creatine phosphate is largely resynthesized in frog muscle before any considerable loss of lactate or hydrogen ions occurs (Mainwood *et al.* 1972). The intracellular lactic acid would increase to about 0·5% or 10^{-4} M under these conditions. It is possible that even higher concentrations may be found at the inner surface of the membrane due to the influence of fixed charges on the pH of the interface (Pethica & Betts, 1956). In spite of this, it is clear that extracellular lactic acid must be maintained at a very low level for any diffusion gradient to exist. To do this the hydrogen ion concentration immediately outside the membrane must be kept low. The external supply of buffer by diffusion may then become the rate limiting step in this dynamic balance.

The recovery of twitch tension following fatigue is generally enhanced by the same conditions that lead to increased net loss of hydrogen ions and high lactate efflux rates. This is true of four of the five buffer systems used. The imidazole buffer system is less effective in restoring twitch tension than the other four buffers, although it supports high lactate efflux rates. This may be related to an additional stimulating effect of imidazole on lactate production since it can be seen that resting lactate efflux levels are considerably higher with imidazole than with any of the other buffer systems used.

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